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Short communication

Staphylococcus aureus intramammary infection elicits increased production of transforming growth factor-α, β1, and β2

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Abstract

In contrast to other mastitis pathogens, the host response evoked during *Staphylococcus aureus* intramammary infection is marked by the absence of the induction of critical cytokines, including IL-8 and TNF-α, which have established roles in mediating host innate immunity. The elucidation of changes in the expression of other mediators with the potential to regulate mammary inflammatory responses to *S. aureus* remains lacking. Transforming growth factor (TGF)-α, TGF-β1, and TGF-β2 are cytokines that regulate mammary gland development. Because these cytokines also have a demonstrated role in mediating inflammation, the objective of the current study was to determine whether *S. aureus* intramammary infection influences their expression. Ten cows were challenged with *S. aureus* and milk samples collected. Increases in milk levels of TGF-α were evident within 32 h of infection and persisted for 16 h. Increases in TGF-β1 and TGF-β2 levels were detected within 40 h of *S. aureus* infection and persisted through the end of the study. Thus, in contrast to IL-8 and TNF-α, *S. aureus* elicits host production of TGF-α, TGF-β1, and TGF-β2. This finding may suggest a role for these cytokines in mediating mammary gland host innate immune responses to *S. aureus*.
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Keywords: Cytokine; *Staphylococcus aureus*; Mammary gland; Mastitis; Transforming growth factor

1. Introduction

The innate immune system represents the first line of active defense in the host response to infection. Soluble proteins known as cytokines, which mediate the host inflammatory response to infection, are a critical component of the innate immune system. For example, IL-1β, IL-8, and TNF-α are cytokines that promote inflammation by

Abbreviations: PGE2, prostaglandin E2; SCC, somatic cell count; S.E., standard error

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altering vascular permeability, promoting leukocyte recruitment, eliciting a febrile response, and inducing hepatic synthesis of proteins that facilitate bacterial recognition and complement activation (Feghali and Wright, 1997). Another cytokine with pro-inflammatory properties is TGF-α (Derynck, 1992). This cytokine is expressed by innate immune effector cells (e.g., neutrophils, macrophages, and eosinophils), as well as other cell types, including epithelial cells and fibroblasts (Calafat et al., 1997). Consistent with a pro-inflammatory role, TGF-α upregulates IL-8 and prostaglandin E_2 (PGE_2) production and synergistically enhances the effects of IL-1β and TNF-α (Bry, 1993; Subauste and Proud, 2001). TGF-α further contributes to the induction of host defense mechanisms by promoting the expression of anti-microbial peptides (Sorensen et al., 2003).

Since prolonged inflammation can result in tissue damage, induction of a counter-regulatory anti-inflammatory response is necessary to limit injury to host tissues. One such cytokine, IL-10, promotes resolution of inflammation by downregulating pro-inflammatory cytokine production (Spits and de Waal Malefyt, 1992). Other cytokines implicated in limiting the scope of inflammation are members of the TGF-β family, which act on macrophages and other cell types to inhibit pro-inflammatory responses and enhance removal of bacterial debris, inflammatory cells, and injured parenchymal cells (Letterio and Roberts, 1998; Ashcroft, 1999).

Establishment of infection is governed, in part, by the nature of the host response to the invading organism (Bannerman et al., 2004). It has been reported that intramammary infection by *Escherichia coli* often follows a distinct clinical course compared to that of *Staphylococcus aureus*. Intramammary infection by *E. coli* is acute in nature and generally clears within a few days (Smith and Hogan, 1993). In contrast, intramammary infection by *S. aureus* is often less acute, but results in a chronic infection that can persist for the life of the animal (Sutra and Poutrel, 1994). We and others have established that the differential inflammatory response elicited during *E. coli* and *S. aureus* intramammary infection corresponds with the outcome of infection (Riollet et al., 2000; Bannerman et al., 2004). Compared with *S. aureus*, intramammary infection by *E. coli* elicits an acute and more pronounced febrile response, prolonged increases in mammary vascular permeability, and higher levels of complement activation and production of IL-8, IL-10, and TNF-α. Of particular note, *S. aureus* intramammary infection fails to elicit any detectable production of IL-8 or TNF-α. Together, these data demonstrate the variability of the host innate immune response to intramammary pathogens, and suggest that a limited inflammatory response may contribute to the development of chronic intramammary infection.

Although much is known about the expression of the classic pro-inflammatory cytokines IL-1β, IL-8, and TNF-α during the innate immune response to various mastitis pathogens (Riollet et al., 2000; Bannerman et al., 2004), much less is known about the expression of cytokines such as TGF-α, TGF-β1, and TGF-β2, which have a postulated role in regulating inflammatory processes during the course of this disease. Recently, we have reported that *E. coli* intramammary infection elicits the expression of these three cytokines (Chockalingam et al., 2005). Because the production of other cytokines are known to be differentially induced by *E. coli* and *S. aureus* (Riollet et al., 2000; Bannerman et al., 2004), we investigated whether intramammary infection with *S. aureus* could similarly upregulate expression of TGF-α, TGF-β1, and TGF-β2.

2. Materials and methods

2.1. Animals

Ten healthy, mid-lactating Holstein cows (188 ± 16 days in milk) free of intramammary infection were selected for the study. The mean (±S.E.) milk somatic cell counts (SCC) in control and challenged quarters prior to infection was 54,920 ± 26,792 cells/ml. The use and care of all animals in this study were approved by the Beltsville Agricultural Research Center's Animal Care and Use Committee.

2.2. Intramammary challenge with S. aureus

S. aureus Newbould strain 305 (American Type Culture Collection, Manassas, VA), which was originally isolated from a clinical case of mastitis,

was cultured and prepared for experimental intramammary inoculation as previously described (Bannerman et al., 2004). Immediately following the morning milking, contralateral quarters were infused with either sterile PBS or 67 CFU of *S. aureus* suspended in PBS. Following challenge, aseptic milk samples were collected from infused quarters at various time points, serially diluted, and plated on blood agar plates. After an overnight incubation at 37 °C, colonies were enumerated. Colonies displaying hemolysis were initially counted as *S. aureus*, and subsequently confirmed microscopically and biochemically by the presence of Gram-positive cocci that were both catalase- and coagulase-positive.

2.3. ELISA's and determination of milk somatic cell counts (SCC)

Quantification of milk levels of BSA, IL-8, and TNF-α by ELISA and the enumeration of milk SCC were performed as previously described (Bannerman et al., 2004). Milk levels of TGF-α, TGF-β1, and TGF-β2 were quantified using commercially available kits (R&D Systems Inc., Minneapolis, MN) that have been previously validated for use with bovine milk samples (Ginjala and Pakkanen, 1998; Pakkanen, 1998; Chockalingam et al., 2005). For the determination of milk TGF-α concentrations, undiluted whey samples were directly analyzed. Samples assayed for TGF-β1 were first activated by incubating undiluted whey with an equal volume of an aqueous solution containing 2.5 N acetic acid and 10 M urea for 10 min. The reaction was then neutralized by the addition of a half volume of an aqueous solution containing 2.7 N sodium hydroxide and 1 M HEPES, and the final reactants diluted four-fold with the supplied diluent. Samples assayed for TGF-β2 were first diluted (1:13) in deionized water and subsequently activated according to the manufacturer's instructions.

2.4. Statistical methods

Repeated measures ANOVA was performed using the PROC MIXED model (SAS 8.2; SAS Institute, Cary, NC) to compare the mean responses between experimental groups and the pre-infused (time 0) groups. For statistical analysis of milk SCC, data were transformed to $\log_{10}$ values. An unpaired *t*-test (GraphPad Prism version 4.0 for Windows; GraphPad Software Inc., San Diego, CA) was used to compare the maximal responses elicited by *S. aureus* and those reported previously for *E. coli* (Chockalingam et al., 2005). A *P*-value of <0.05 was considered significant.

3. Results and discussion

3.1. Intramammary S. aureus growth following experimental infection

Within 16 h of infusion, *S. aureus* were recovered from the milk of all 10 experimentally infected quarters (data not shown). Transient changes were observed throughout the study in the percentage of quarters from which viable *S. aureus* were recovered, however, at the final sampling 168 h post-infection, 8 of the 10 quarters were still infected. *S. aureus* counts in milk peaked at 24 h post-infection (4.38 ± 0.11 $\log_{10}$ CFU/ml) and remained relatively constant for the duration of the experiment. Saline-infused quarters remained free of infection throughout the study.

3.2. Systemic and localized inflammatory responses to S. aureus intramammary infection

As an indicator of a systemic response to *S. aureus* infection, rectal temperatures were monitored throughout the study. A trend in elevation of body temperature was apparent from 8 to 40 h post-infection, however, statistically significant increases were only observed at the 8 and 32 h time points (data not shown). Maximal elevations in temperature were detected 32 h after infection and reached a peak mean (±S.E.) of 39.23 ± 0.15 °C.

Changes in milk SCC were monitored throughout the study as an indicator of local inflammation (Fig. 1A). Within 24 h of infection, initial increases in milk SCC were observed in *S. aureus*-infused quarters and elevated levels of somatic cells persisted for the duration of the experiment. Relative to pre-infused (time 0) levels, milk SCC in quarters infused with saline remained unchanged throughout the study.

Changes in milk BSA levels, which reflect alterations in mammary vascular permeability, were also monitored as another indicator of local inflam-

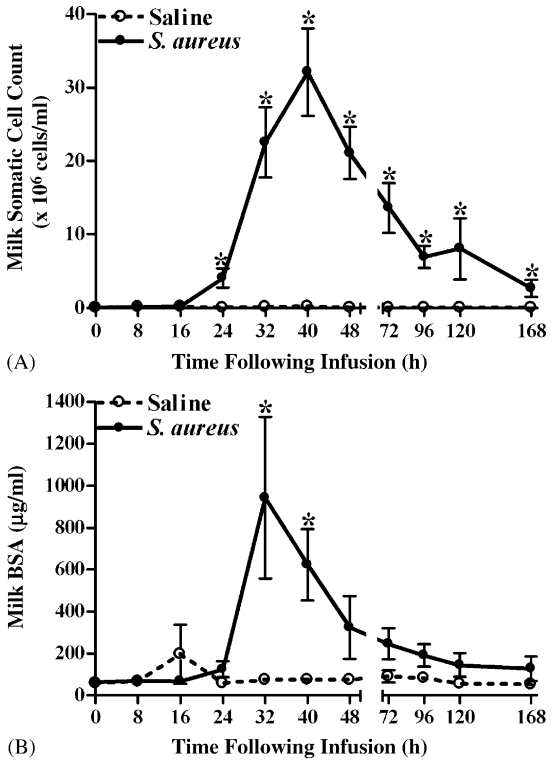

Fig. 1. Effect of *S. aureus* intramammary infection on milk somatic cell counts (SCC) and mammary vascular permeability. Milk SCC (A) and bovine serum albumin (BSA) (B), the latter of which is an indicator of changes in the blood-mammary gland barrier, were quantified in milk samples collected from both phosphate-buffered saline control and *S. aureus*-infected quarters. Mean (±S.E.) levels are reported. *Significantly increased relative to time 0 ($P < 0.05$).

mation (Fig. 1B). Increased milk levels of BSA were detected in *S. aureus*-infected glands within 32 h of infusion and persisted for an additional 8 h. Maximal mean (±S.E.) levels of BSA in infected quarters reached 941.75 + 385.96 μg/ml. Milk BSA concentrations in saline-infused quarters remained unchanged throughout the study.

As a final indicator of inflammation, milk samples obtained from saline- and *S. aureus*-infused quarters were assayed by ELISA for the induction of the pro-inflammatory cytokines, IL-8 and TNF-α. Positive control whey samples from previous studies were assayed in parallel. Consistent with previous reports (Riollet et al., 2000; Bannerman et al., 2004), there was no detectable induction of either cytokine in *S. aureus*-infected or saline-infused quarters at any time point throughout the study (data not shown).

3.3. Elevations in milk TGF-α concentrations during intramammary S. aureus infection

TGF-α levels were detectable in the milk of all quarters prior to infusion (time 0) (Fig. 2A). There were no significant differences ($P = 0.73$) in the basal (time 0) milk levels of TGF-α in quarters subsequently infused with saline (54.40 ± 21.53 pg/ml) versus those infused with *S. aureus* (45.30 ± 15.82 pg/ml). Within 32 h of challenge, an increase in milk TGF-α levels above pre-infused (time 0) levels was evident in *S. aureus*-infected quarters and this increase was sustained for an additional 16 h. Peak levels were detected 40 h post-infection and reached a mean (±S.E.) maximum concentration of 355.39 ± 70.00 pg/ml. In quarters infused with saline, TGF-α milk levels remained unchanged throughout the study relative to those detected prior to infusion.

Recently, TGF-α production has been reported to be upregulated during *E. coli* intramammary infection (Chockalingam et al., 2005). The current study establishes that *S. aureus* similarly evokes the upregulation of TGF-α expression (Fig. 2A). Peak elevations in TGF-α levels elicited by infection with *S. aureus* (355.39 ± 70.00 pg/ml) were not statistically different ($P = 0.25$) than those reported during *E. coli* infection (500.53 ± 98.41 pg/ml) (Chockalingam et al., 2005). However, the lag time between infection and initial detection of increased TGF-α was longer following *S. aureus* infection, and the duration of sustained increases shorter, relative to that observed following *E. coli* infection. The abridged elevation of TGF-α in *S. aureus*-infected glands is consistent with the reported diminished pro-inflammatory response profile elicited by *S. aureus* versus that evoked by *E. coli* (Riollet et al., 2000; Bannerman et al., 2004).

The patho-physiological effect of increased TGF-α in the gland during *S. aureus* infection remains unknown. TGF-α has been reported to synergistically enhance the effects of IL-1β and TNF-α (Bry, 1993; Subauste and Proud, 2001). IL-1β, but not TNF-α, is upregulated during *S. aureus* intramammary infection (Bannerman et al., 2004). Thus, in the absence of TNF-α, the ability of the host to mount an inflammatory response to *S. aureus* may be even more critically dependent on TGF-α enhancement of IL-1β's pro-inflammatory effects. TGF-α has also been reported to disrupt mammary epithelial tight junction formation

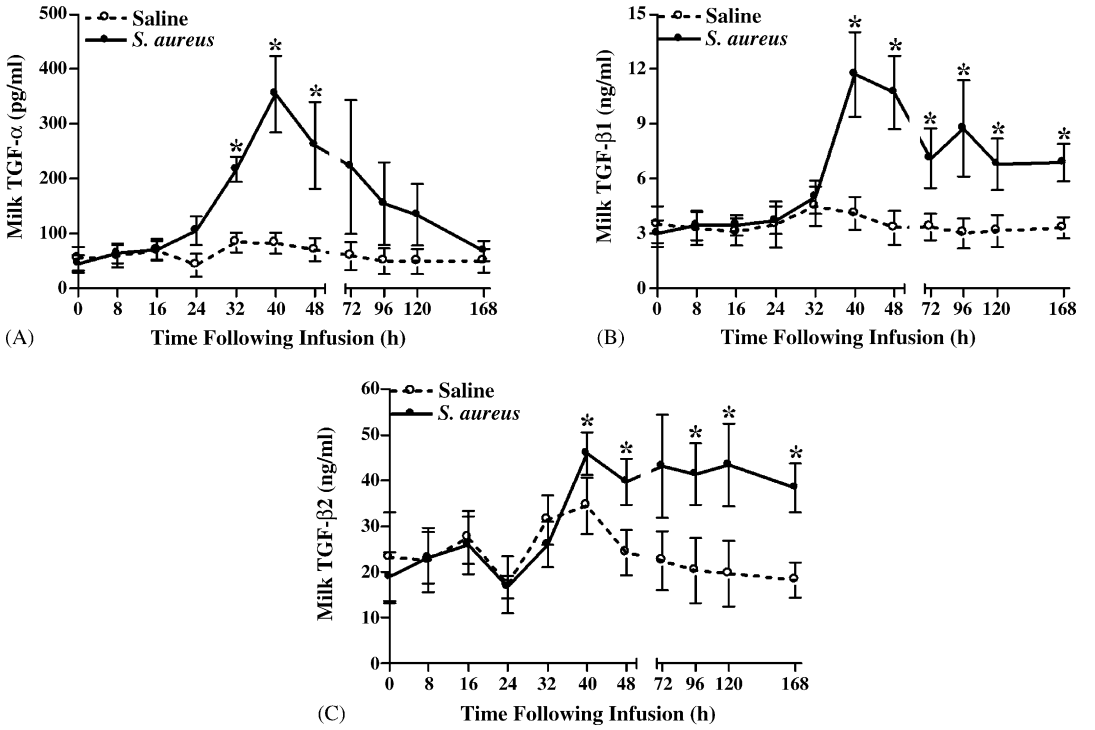

Fig. 2. Effect of intramammary inoculation with *S. aureus* on TGF-α, TGF-β1, and TGF-β2 concentrations in milk. Following intramammary infusion of either phosphate-buffered saline or *S. aureus*, milk samples were collected at various time points and milk TGF-α (A), TGF-β1 (B), and TGF-β2 (C) concentrations assayed by ELISA. Mean (±S.E.) concentrations are reported. *Significantly increased relative to time 0 ($P < 0.05$).

(Buse et al., 1995) and to elicit ascites formation (Ohmura et al., 1990). Consistent with a potential role for TGF-α in influencing mammary vascular permeability, initial increases in TGF-α (Fig. 2A) were temporally coincident with initial increases in BSA (Fig. 1B) and changes in TGF-α levels correlated ($r = 0.72$; $P = 0.01$) with those of BSA. Finally, TGF-α is well established to promote tissue repair, mammary epithelial proliferation, and mammary gland morphogenesis (Derynck, 1992). Since injury to the epithelial lining of the gland is an often deleterious outcome of the inflammation that accompanies mastitis, TGF-α may both promote inflammation and contribute to the resolution of its effects.

3.4. Changes in milk concentrations of TGF-β1 and TGF-β2 during S. aureus intramammary infection

Detectable levels of TGF-β1 and TGF-β2 were present in milk under basal conditions prior to challenge (time 0) (Fig. 2B and C). Equivalent levels of TGF-β1 ($P = 0.68$) were detected in milk at time 0 in quarters subsequently infused with saline (3.50 ± 0.99 ng/ml) compared with those subsequently infused with *S. aureus* (3.00 ± 0.72 ng/ml). Similarly, there were no significant differences ($P = 0.69$) in initial (time 0) milk TGF-β2 concentrations in quarters subsequently challenged with either saline or *S. aureus* (23.14 ± 9.95 ng/ml versus 18.92 ± 5.30 ng/ml, respectively). Initial increases in TGF-β1 and TGF-β2 levels in milk from *S. aureus*-infused quarters were detected within 40 h of infection and reached mean (±S.E.) maximal concentrations of 11.71 ± 2.32 ng/ml (Fig. 2B) and 45.93 ± 4.71 ng/ml (Fig. 2C), respectively. Elevations in TGF-β1 were sustained throughout the study. With the exception of the 72 h time point at which the highest variation in samples was observed, increased levels of TGF-β2 were similarly sustained for the duration of the experiment. In contrast to *S. aureus*-infected glands, the levels of TGF-β1 and TGF-β2 in milk obtained

from saline infused quarters remained unchanged relative to basal (time 0) concentrations throughout the study.

TGF-β is a pleiotropic cytokine that regulates cell growth and differentiation, as well as, inflammatory responses (Letterio and Roberts, 1998; Bonewald, 1999). In the mammary gland, TGF-β regulates ductal growth and alveolar development via its inhibitory effect on epithelial cell growth and its stimulatory effect on fibroblasts and other stromal cells (Daniel et al., 2001). In regard to host responses to infection, TGF-β is best characterized for its role in suppressing immune and inflammatory responses although some pro-inflammatory properties have been ascribed to this molecule (Letterio and Roberts, 1998; Ashcroft, 1999). Whether TGF-β functions as an activator or suppressor of inflammation is dependent upon the location and activation state of the cells that it is stimulating and the presence of other cytokines. Macrophages are a key target of this cytokine and TGF-β has been reported to inhibit their production of chemokines, pro-inflammatory cytokines, nitric oxide, and reactive oxygen intermediates (Letterio and Roberts, 1998; Ashcroft, 1999). In addition, TGF-β inhibits mast cell and T cell activation and function (Cerwenka and Swain, 1999; Gomez et al., 2005). Further supporting an anti-inflammatory role for this cytokine are studies reporting that exogenous administration of TGF-β suppresses inflammation in a variety of disease states (Johns et al., 1991; Santambrogio et al., 1993; Neurath et al., 1996; Powrie et al., 1996; Williams et al., 2005).

The finding that recombinant TGF-β inhibits bovine milk mononuclear cell activation and IL-2 production (Ayoub and Yang, 1997) demonstrates the ability of this cytokine to regulate inflammation and host immune responses in the mammary gland. There are three known mammalian isoforms of TGF-β, however, only two, TGF-β1 and TGF-β2, have been detected in bovine milk (Jin et al., 1991; Ginjala and Pakkanen, 1998). The concentrations of TGF-β1 (3.23 ± 0.58 ng/ml) and TGF-β2 (20.79 ± 5.16 ng/ml) detected in bovine milk under basal conditions (time 0) in the current study are comparable with those reported previously (Ginjala and Pakkanen, 1998; Pakkanen, 1998; Chockalingam et al., 2005). The finding that TGF-β2 is the predominant form expressed in milk is also consistent with prior findings (Jin et al., 1991; Chockalingam et al., 2005).

Similar to *E. coli* (Chockalingam et al., 2005), *S. aureus* intramammary infection evoked increased production of both TGF-β1 and TGF-β2. Maximal concentrations of TGF-β1 detected during the course of *S. aureus* (11.71 ± 2.32 ng/ml) (Fig. 2B) and *E. coli* (13.27 ± 1.94 ng/ml) (Chockalingam et al., 2005) infection were comparable ($P = 0.61$). The higher maximal levels of TGF-β2 detected in the milk of *E. coli*-infected animals (72.99 ± 16.09 ng/ml) (Chockalingam et al., 2005) did not statistically differ ($P = 0.14$) from those detected in *S. aureus* infected quarters (45.93 ± 4.71 ng/ml). In the current study, indicators of inflammation, including elevations in temperature, milk SCC (Fig. 1A), mammary vascular permeability (Fig. 1B), and TGF-α production (Fig. 2A), were all evident prior to detectable increases in TGF-β, a finding similar to that observed during *E. coli* intramammary infection (Chockalingam et al., 2005). Thus, consistent with a potential counter-regulatory, anti-inflammatory role for TGF-β, its induction occurs downstream of initial pro-inflammatory responses.

The current study is the first to investigate changes in TGF-α, TGF-β1, and TGF-β2 during the course of intramammary infection with *S. aureus*. Although a previous study reported on changes in the expression of these cytokines in the context of experimental *E. coli* mastitis (Chockalingam et al., 2005), extrapolation of those findings to *S. aureus* is difficult due to the distinct cytokine responses elicited by these two pathogens in vivo (Riollet et al., 2000; Bannerman et al., 2004). This study clearly establishes that *S. aureus* is able to evoke increased production of TGF-α, TGF-β1, and TGF-β2 during the course of intramammary infection. Further studies will be needed to identify the direct role that these cytokines have on the outcome of intramammary infection caused by this and other mastitis pathogens.

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